MICROBIOLOGY TECHNOLOGY REPORT

Oral Biofilm Inhibiting Properties Of CPC And Stannous Fluoride

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BACKGROUND

Bacterial organisms are capable of binding to and "colonizing" a wide range of surfaces. Bacterial "colonies" established on a surface are called biofilms, and are evident in a wide variety of situations such as the hull surface of ships, the metallic surfaces in air conditioning units, and even rock surfaces on the bottom of swiftly running streams. Of interest to the healthcare community is the ability of bacterial organisms to colonize plastic surfaces (the interior of catheters), and even surfaces and tissues associated with the human body itself. The plaque that builds up on teeth is a good example of the latter. At first, a few organisms attach to the tooth surface. When a certain number of bacteria are attached (i.e. reaching a "quorum"), the organisms communicate with each other and begin producing an extracellular matrix (ECM) that surrounds the organisms. Once this ECM is established, it offers significant protection from antibiotics and other substances that may be detrimental to the bacteria. The organisms that are buried deeply in the biofilm simply cannot be reached. When considering the oral cavity, these biofilms present a significant problem due to the fact that the organisms contained within often contribute to oral malodor and produce virulence factors involved in gum disease.

In light of the importance of biofilms in the oral cavity, P&G is interested in the effects of currently available oral care products along with various upstream technologies on biofilm growth. Two actives that are currently under evaluation are cetylpyridinium chloride (CPC), and stannous fluoride (SnF_2).

CPC is a quaternary ammonium compound that has a long aliphatic chain (C16). Its antimicrobial activity comes from the fact that the molecule has both a positively charged (hydrophilic) and a hydrophobic region. The positively charged head is attracted to the outer surface of the cell membrane, and then the hydrophobic tail inserts into the membrane. This process allows the leakage of intracellular components which leads to cell death[1]. P&G has a currently marketed oral rinse containing 0.05% CPC (Scope), however, the bioavailability associated with this rinse is quite low (150ppm) due to the presence of surfactants. The formulation tested in the assays described below were re-formulated to give a much higher bioavailability.

Stannous fluoride was the source of fluoride in the original Crest dentifrice that first received the ADA seal of approval for caries prevention. In addition to forming a protective coat on the tooth ($Sn_3F_3PO_4$), it has been demonstrated that stannous also has an associated antimicrobial effect. Bacteria exposed to SnF_2 retain large quantities of the tin species intracellularly. It has been suggested that stannous inhibits growth by oxidizing thiol groups in critical enzymes (described below). In addition, stannous concentrations as low as 0.001% have been shown to inhibit the coherence of organisms such as *S. mutans*[2].

OBJECTIVE

The objective of the two studies described below was to investigate the ability of stannous and CPC to inhibit the growth of an *in-vitro* oral biofilm using the plaque chip model. The studies were conducted using human saliva, and the data described here was obtained from two separate studies.

APPARATUS
Anaerobic Incubator set at 36°C
Vortex Mixer
Autoclaves
Spiral Biotech Autoplate 4000 spiral plater
Biosafety cabinet
Spiral Biotech Q Count automated colony counting system

MEDIA
Enriched tryptic soy agar (ETSA; Total Anaerobes), 100 x 15 mm plates
Enriched tryptic soy agar with nalidixic acid and vancomycin (total gram-negative Anaerobes), 100 x 15mm plates
Crystal violet media (CV; selective for *Fusobacteria*), 100 x 15mm.

REAGENTS AND SUSPENSION MEDIA
D/E Neutralizing broth: Difco Brand.
Sterile Water

MATERIALS
Gilson Pipettors, 100 - 1000µl capacity
Sterile Pipet Tips, 200 µl,
Gloves, Latex, Powder-Free
30% bleach
Paper Towels
Synthetic enamel chips
Sterile Cryovials (2ml size)

TEST PRODUCTS
High Bioavailable CPC (HBA-CPC) rinse
Crest Gum Care dentifrice

TEST PROCEDURE

The studies described in this report were conducted as follows. The Plaque chip model is an *in vitro* assay that uses a synthetic enamel (synamel) chip as a surface on which a saliva based biofilm is grown. Both of these assays ran for four days. To start the assay, a set of two milliliter sterile cryovials were used, and the synthetic enamel chips were mounted to the inside of the caps (Figure #1). The chips were held in place with a small bit of wax. Saliva was collected from a minimum of four donors, pooled, and mixed to homogeneity. Enough 10% sucrose in water solution was added to give the saliva a 0.1% sucrose concentration. One milliliter of this saliva was then aseptically aliquoted out into each of the sterile cryovials to be used in the assay. The caps containing the chips were threaded onto the tubes, and they were then placed into a holding box. The vials were taken into a monitored warm room which is held at 33°C and 60% relative humidity, and placed on a rotator (Figure #3) at 1rpm. This allows the saliva to use the added sugar to get a biofilm (plaque) started on the enamel surface. The vials were rotated for six hours. Following the six hour incubation, fresh saliva was collected, pooled, and mixed to homogeneity. It was aliquoted out (in 1ml quantities) into enough cryovials to provide one for each chip. At this point the treatments began.

In an attempt to simulate the application of a product in the oral environment, both actives were applied in the form of a 1ml treatment solution for 1 min. A second set of tubes was created containing 1ml aliquots of the two treatment solutions. The caps with the beginning plaque accumulation was placed onto them. They were attached to a rotator on the bench top (Figure #4), and rotated at 10rpm for 1 minute. The chips were then transferred to the set of tubes with fresh saliva (without sucrose), and placed back on the rotator in the warm room. The chips were allowed to rotate in the warm room overnight until the morning of the second day.

On the second day, saliva collection, treatment, and incubation were carried out just as they were on the evening of the first day. However, it was done twice (once in the morning and once in the afternoon.). The same thing occurred on the third day.

The morning of the fourth day was when the plaque was collected and plated. Enough empty tubes were placed into a rack to provide one tube for each chip in the assay. Then approximately 10-15 glass beads were placed into each tube. One milliliter of sterile water was then transferred into each with a pipette. The chips were retrieved from the rotator in the warm room. Each chip was placed onto a tube with saline/glass beads in it. The rack with the chips and glass bead/sterile saline tubes was then inverted and clamped into a multitube vortexer (figure #6), and vortexed for 1 minute. This proved to be an effective method for removing the plaque from the chips in preparation for plating.

Next, the plaque containing solutions had to be diluted because they were too many bacteria to plate. For the assays described here, the plaque slurries were diluted out to 1:2000. This produced plates with no crowding, but enough colonies such that no low counts (less than 30 colonies) were obtained.

Since the plaque was knocked off of the chip using 1ml of saline, it was virtually impossible to get a full 1milliliter of sample to do a standard 1:10 dilution of the plaque. Because of this, a 0.5ml sample was taken and added to a tube containing 9.5ml of D/E broth. This produced a 1:20 dilution. The 1:20 dilution was vortexed, and then 1ml was placed into the 1:200 tube which had 9ml of D/E in it. The same step was repeated again to obtain the final dilution of 1:2000.
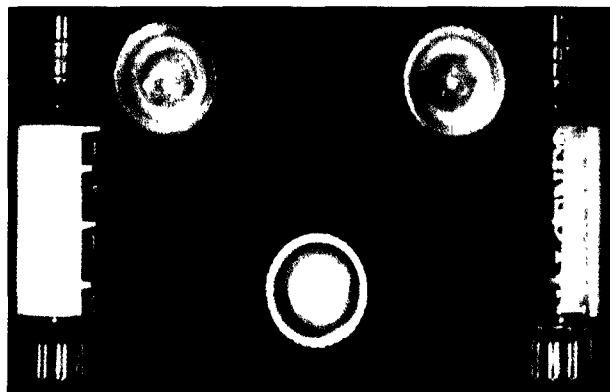

Figre #1. Vials used in the plaque chip assay.
The two caps at the top have no synthetic chip in them, and the bottom one does.
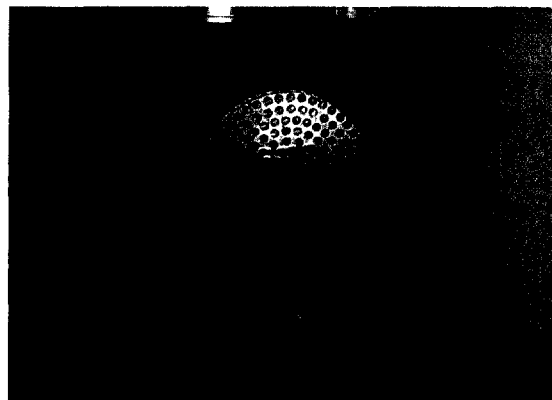
Figure #3. Rotator in the monitored warm room. A set of tubes is shown attached for rotation/incubation.
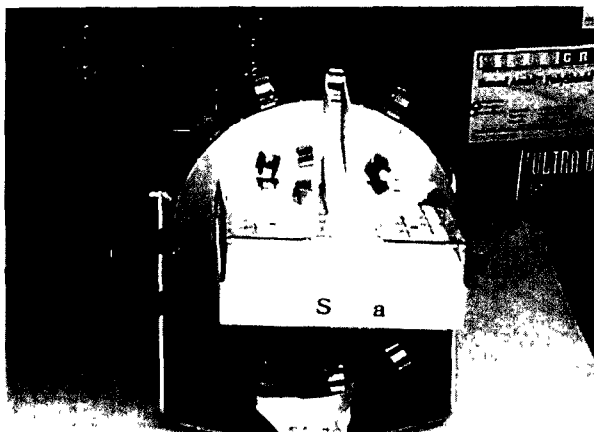
Figure #4. This figure shows a rack of vials Mounted to a rotator on the bench top. This set-up is used for exposing the chips to their respect-ive treatment solutions.
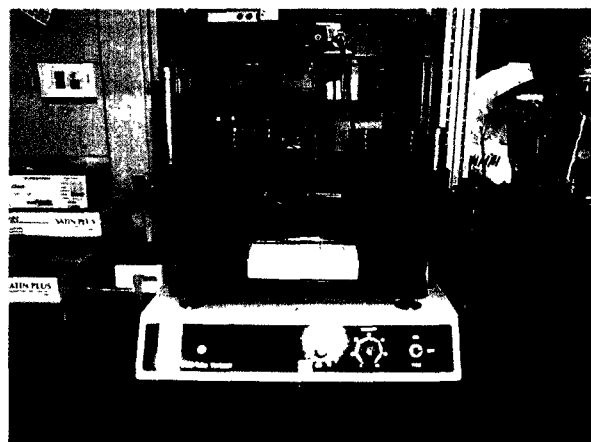
Figure #6. The tubes are vortexed for 1 minute to Remove the plaque.

Once the samples were diluted, the plating media was selected. Two different media were used, and they are outlined below.

- Enriched Tryptic Soy Agar (ETSA): This media contains whole, lysed sheep blood. It is purchased from Anaerobe Systems Inc. It is commonly used to obtain a count of the total facultative anaerobes present in a sample.

- Enriched Tryptic Soy Agar with Nalidixic Acid and Vancomycin (ETSA-NV): As with the ETSA plates, this media contains whole, lysed sheep blood. And it is produced anaerobically by the above company. However, it also contains nalidixic acid and vancomycin. These two compounds significantly suppress gram positive organisms. This media is used to obtain total facultative Gram-negative anaerobe counts from samples.

Once the media was selected, the samples were ready to be plated. This was accomplished using a Spiral Biotech Autoplate model 4000. The autoplater was set up to plate an increasingly dilute concentration of the sample as it moved from the center of the plate out to the edge. For a detailed description, see the spiral plater manual.

After all of the samples were plated, they were stacked into holding racks and placed into an incubator that is contained within a Coy brand anaerobic chamber. They were allowed to incubate for 72 hours at 36°C, and then were removed for counting.

After the plates were removed from the incubator, the colonies on each needed to be counted in order to calculate the CFU/ml contained in the original plaque samples. This was accomplished using the Spiral Biotech Q Count imaging based colony counting system (Figures 7 and 9 below). Before counting began, a few decisions were made for the parameters associated with the instrument. These parameters maximize the contrast between the colonies and their background (i.e. the media surface). For the software, the shutter speed has to be selected (between 125 and 1000), and you must choose between bottom lighting and top lighting. The optimal settings used for each of the media in the assays described here are given below

- For the ETSA and ETSA-NV media : The shutter speed is set to 500, bottom lighting is used, and no filter is put in place.
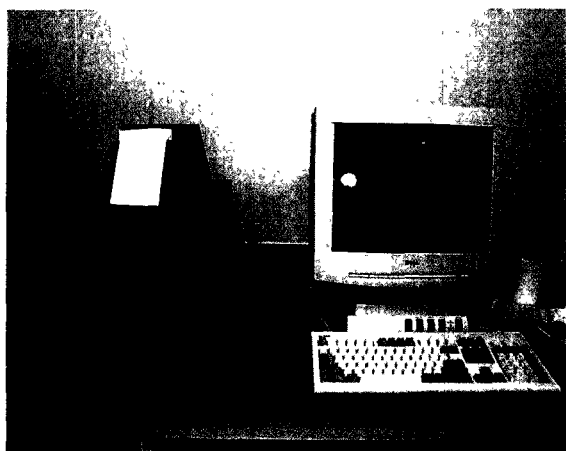
Figure #7. This is a photograph of the entire Q Count system.
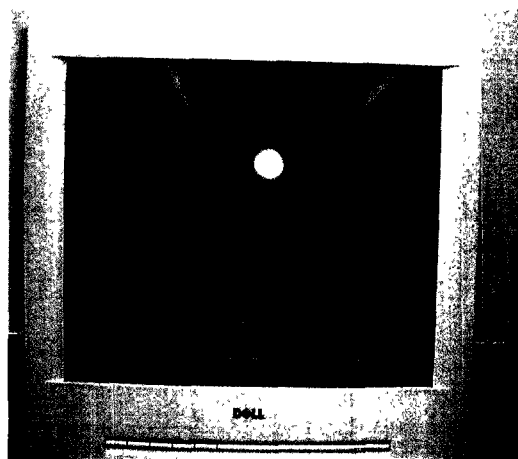
Figure #9. This is a close up of the screen for the Q Count. It is difficult to see, but there are green dots on the screen representing the colonies.

Once the data was completely collected by the Q count, the file was saved in an Excel format. The CFU/ml data along with all of the settings used was given. The data was then manipulated for presentation as shown below.

RESULTS

1. **Table 1** gives a summary of all the *in-vitro* CPC data relevant to this report. The table is sorted by type of bacteria measured and by assay number.

TABLE 1: CPC Effects On Two Different Oral Bacterial Populations Contained Within An *In-Vitro* Oral Biofilm

	Mean Log_{10} CFU/ml Difference Vs. Water		
Oral Flora Sub-Group	Assay #1[a]	Assay #2[a]	Average
Total Facultative Anaerobes	0.84[b]	0.66[b]	0.75
Total Facultative Gram-Negative Anaerobes	0.68[b]	0.88[b]	0.78

[a] All data shown here were calculated by subtracting the mean log CFU/ml for the CPC group from that of the water group.
[b] For all treatments, the mean differences from water were significant with $p < 0.05$.
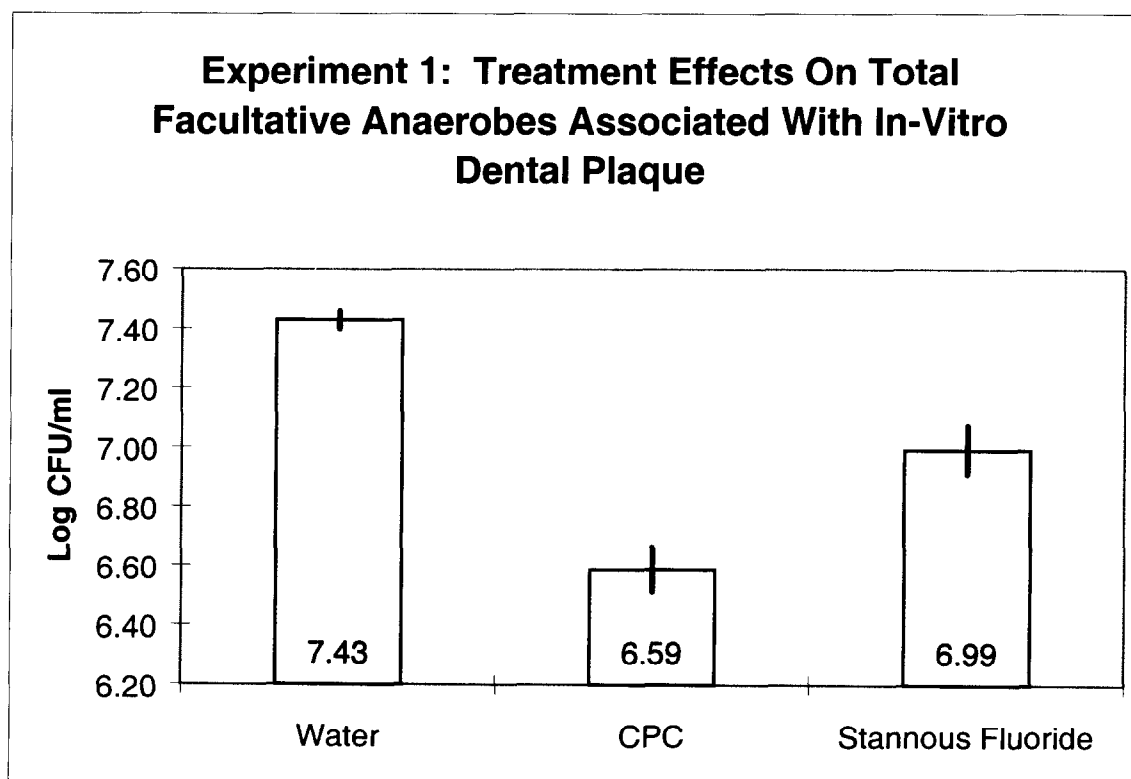
2. **Table 2** gives a summary of all the *in-vitro* stannous fluoride data relevant to this report. The table is sorted by type of bacteria measured and by assay number.
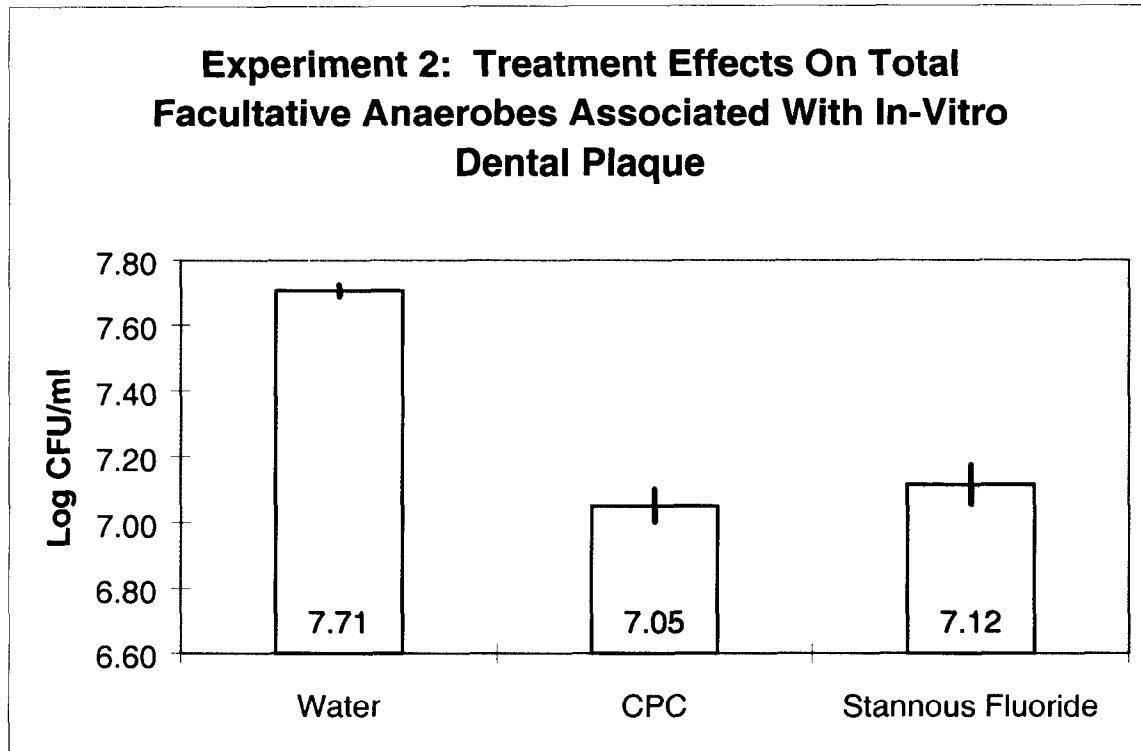
TABLE 2: Stannous Fluoride Effects On Two Different Oral Bacterial Populations Contained Within An *In-Vitro* Oral Biofilm

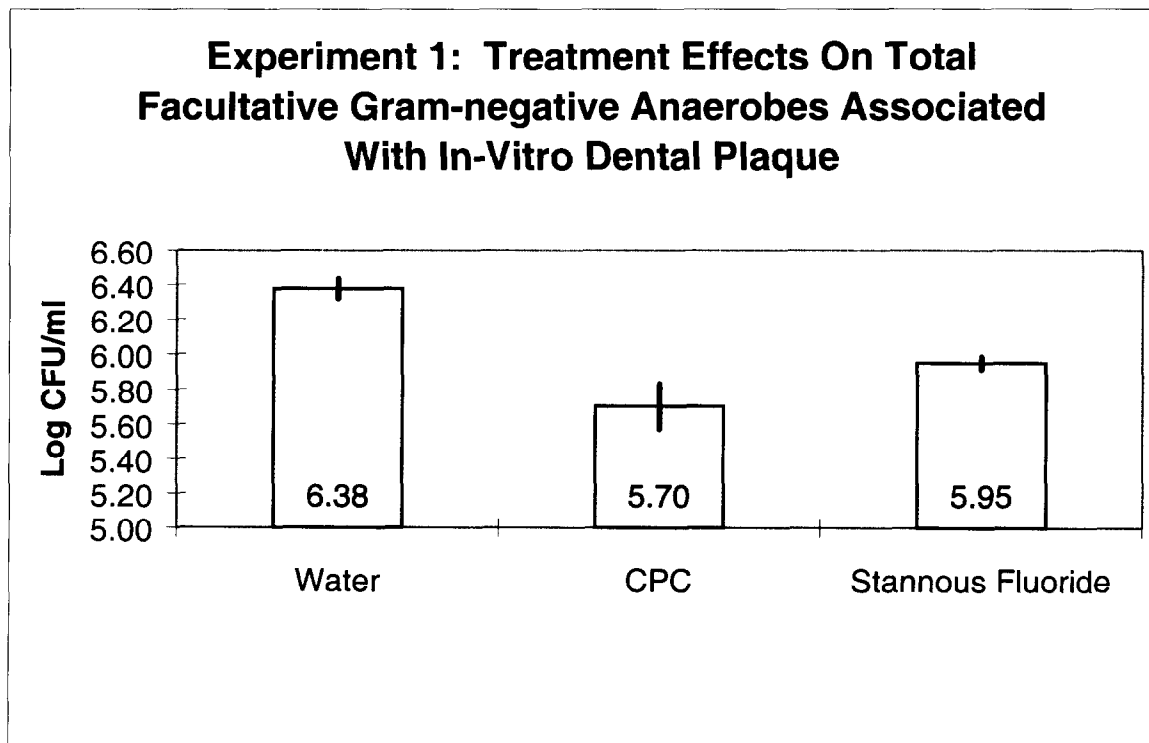|	Mean Log_{10} CFU/ml Difference Vs. Water		
Data Type	**Assay #1[a]**	**Assay #2[a]**	**Average**
Total Facultative Anaerobes	0.44^{b}	0.61^{b}	0.52
Total Facultative Gram-Negative Anaerobes	0.43^{b}	0.56^{b}	0.49
[a]All data shown here were calculated by subtracting the mean log CFU/ml for the CPC group from that of the water group. [b]For all treatments, the mean differences from water were significant with $p<0.05$.			

3. The following 2 graphs represent all of the total facultative anaerobe data gathered from the two relevant assays for both CPC and stannous.

Experiment 1: Treatment Effects On Total Facultative Anaerobes Associated With In-Vitro Dental Plaque

Experiment 2: Treatment Effects On Total Facultative Anaerobes Associated With In-Vitro Dental Plaque

4. The following 2 graphs represent all of the total facultative gram-negative anaerobe data

Experiment 1: Treatment Effects On Total Facultative Gram-negative Anaerobes Associated With In-Vitro Dental Plaque

Experiment 2: Treatment Effects On Total Facultative Gram-Negative Anaerobes Associated With In-Vitro Dental Plaque
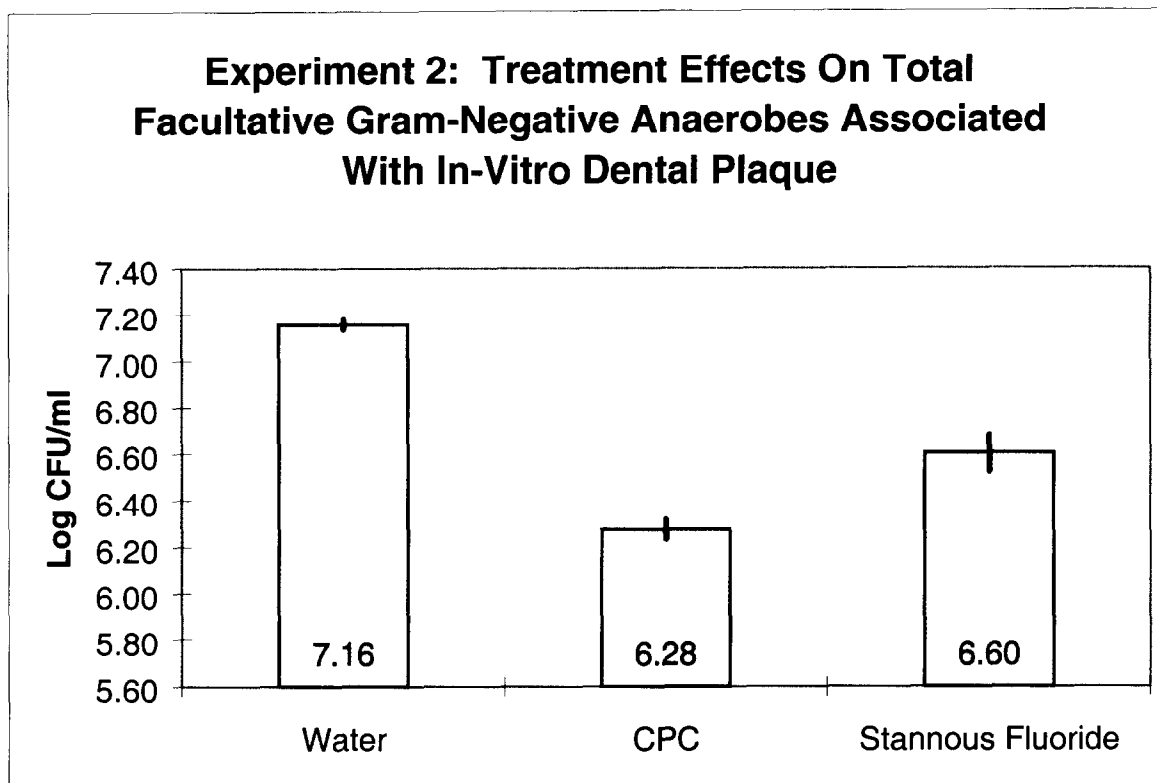
KEY LEARNINGS

- The data in the tables and graphs above pertaining to CPC represent a series of two separate *in vitro* assays examining the ability of CPC to affect the bacterial composition of dental plaque. The mouth rinse formulation used provides approximately 350ppm of bioavailable CPC (0.035%) as measured by the disc retention assay. Treatment for 1 minute twice a day produced an average reduction of the total facultative anaerobes in the plaque chip model of about 0.66 log units. For total facultative Gram negative anaerobes, the reduction versus water was 0.72 log. For both measures, the reduction was significant at $p<0.05$

- The data pertaining to stannous represents a series of two separate *in-vitro* assays examining the ability of stannous to affect the bacterial composition of dental plaque. The stannous fluoride concentration in the actual treatment solution is 0.454%. Treatment for 1 minute twice a day produced an average reduction of the total facultative anaerobes in the plaque chip model of about 0.49 log units. For total facultative Gram negative anaerobes, the difference was 0.69 log units. For both measures, the reduction was significant at $p<0.05$

Discussion CPC.

The data presented above provide strong *in vitro* microbiological evidence that CPC kills the bacteria that cause gingivitis. The assays described above demonstrated that the high bioavailable formulation provided a 0.66 log unit reduction in total facultative anaerobes, and a 0.72 log unit reduction in total facultative Gram-negative anaerobes. This is in contrast to the currently marketed scope formulation which provides only a 0.44 log unit reduction for both groups of organisms in the same assay (data not shown).

The bioavailability of CPC containing rinses is normally evaluated with the disc retention assay. In this assay, a small quantity of the rinse under evaluation is placed onto a cellulose disc, and allowed to sit for 1 minute. The disc is then placed into a vacuum filtration apparatus and Acid fuschin dye is filtered through, followed by several water

rinses to remove excess dye. Any "biovavailable" CPC in the rinse will stick to the cellulose disc, and the dye sticks to the bound CPC. The dye is then rinsed into a para-toluene sulfonic acid (PTSA) solution and measured using a spectrophotometer. A standard curve is created, and from this, the concentration of bioavailable CPC in a rinse can be calculated. Using this technique, the HBA rinse gives a reading of about 350 ppm (0.035%) bioavailability. This is in comparison to a reading of about 150ppm (0.015%) produced by the currently marketed Scope formulation (data not shown).

Finally, one can make inferences about gingival health benefits, based on the antimicrobial/anti-plaque properties of the HBA CPC rinse. Since it reduces plaque accumulation, it would also presumably reduce both the levels of virulence factors expressed by organisms involved in gum disease

Discussion stannous fluoride.

The results reported here provide strong support for the use of stabilized stannous fluoride (accomplished via the use of sodium gluconate as a chelating agent, and stannous chloride as an antioxidant to protect the SnF_2 from oxidation) in a dentifrice to treat and prevent gingivitis. The ability to reduce bacterial numbers associated with dental plaque demonstrates the killing action behind the clinical benefits that have been demonstrated with Crest Gum Care (a currently marketed stannous fluoride dentifrice). These benefits among other things, demonstrated efficacy at reducing gingivitis and gingival bleeding (relative to a sodium fluoride control).[3] This is most likely due, in part, to reduced levels of plaque, and thus reduced levels of the bacterial and host derived virulence factors that contribute to gingivitis.

Stannous fluoride also produces a caries benefit through two distinct mechanisms. The stannous and fluoride provide a protective coat to the tooth (or synamel chip in the case of the plaque chip model) surface as mentioned above in the background section of this report. Secondly, stannous has an inhibitory effect on the metabolism of oral bacteria such that their growth is at the very least attenuated, and most likely eliminated. This is evidenced by the reductions in plaque associated bacteria shown in the assays described in this report. This decrease in metabolism generates less bacterial acid production in the oral biofilm via the glycolysis pathway. The mechanisms by which stannous inhibits the growth of bacteria involves the inhibition of two key enzymes in the glycolysis pathway by stannous, and the inhibition of a third by fluoride ion. This process is illustrated below in figure #1.

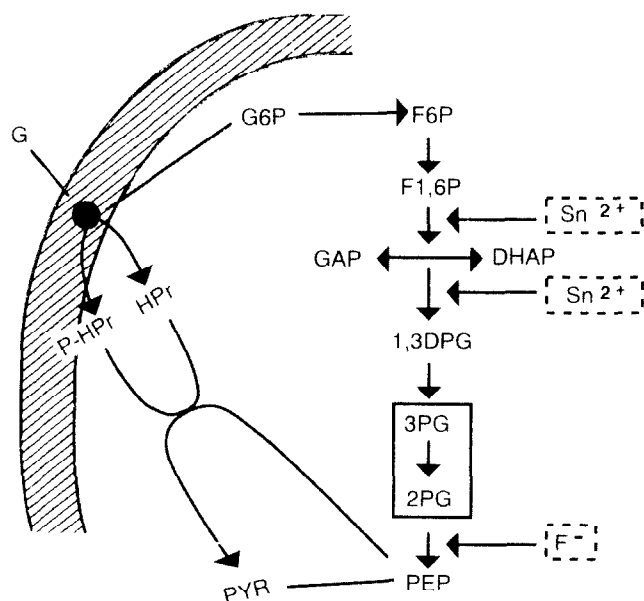### GLYCOLYSIS/F^- and Sn^{2+}

Figure #1 above depicts the mechanism of action for Stannous on enzymes in the glycolysis pathway. Stannous works by inhibiting fructose-1,6-bisphosphate aldolase (the first point in the diagram) and triosephosphate dehydrogenase (the second point in the diagram. The fluoride ion works by inhibiting pyruvate kinase.

In summary, a heavy growth of biofilm on the tooth surface contributes significantly to the clinically proven gingivitis. One recommendation to individuals in this situation would be to improve oral hygiene to reduce plaque levels. However, a second approach for this situation is to devise a dentifrice that not only provides fluoride, but also has an antimicrobial included such that plaque levels are controlled along with the corresponding levels of plaque acids leading to reductions in gingival disease. Based on the *in vitro data* presented above and the clinical data currently available, stannous fluoride containing dentifrices not only reduce and prevent gingivitis, but also kill the bacteria that cause gingivitis..

Conclusion.

The results of the experiments described in this report demonstrate the potent antimicrobial activity of CPC and stannous fluoride against bacteria in a biofilm. Further, the significant reductions in Gram-negative facultative anaerobes provide evidence that CPC and stannous fluoride kill the bacteria that cause gingivitis and form dental plaque.

References

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2. **Tinanoff, N.** 1990. Review of the Antimicrobial Action of Stannous Fluoride. J. Clin. Dent. **2**:22-27.

3. **Beiswanger B.B., P.M. Doyle, R.D. Jackson, M.E. Mallatt, M.S. Mau, B.W. Bollmer, M.M. Crisanti, C.B. Guay, A.C. Lanzalaco, M.F. Lukacovic, S. Majeti, S.F. McClanahan.** 1995. The Clinical Effect of Dentifrices containing Stabilized Stannous Fluoride on Plaque Formation and Gingivitis-A Six-Month Study with *Ad libitum* Brushing. J. Clin. Dent. **6**:46-53